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Heterogeneity of the diverse aerobic sludge granules self-cultivated in a membrane bioreactor with enhanced internal circulation

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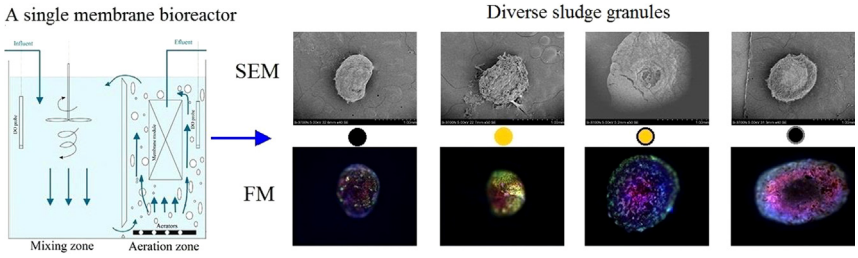
GRAPHICAL ABSTRACT



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ABSTRACT

The present work revealed the heterogeneity of the sludge granules formed in a membrane bioreactor with enhanced internal circulation, and also contributed to better understanding of their forming mechanisms. By continuously carrying out an experiment lasting for more than 3 months with the floc sludge from a local municipal wastewater treatment plant as inoculation sludge, diverse aerobic sludge granules were found to be successfully self-cultivated within the reactor. The results of scanning electron microscopy, fluorescence microscope and high-throughput sequencing measurement indicated that the obtained diverse granules exhibited quite obvious heterogeneity in their basic physico-chemical and microbial properties, and filamentous bacteria actually acted as a main skeleton to keep the self-cultivated sludge granules stable in both their structure and morphology. Furthermore, stable and high COD and TN removal achieved, over 85% and 60%, respectively, which confirmed its usefulness in wastewater treatment.

1. Introduction

Up to date, the main technology adopted for municipal wastewater treatment is the conventional activated sludge (CAS) process. However, numerous theoretic and practical researches claim that a membrane bioreactor (MBR) has advantages in terms of lower footprint, better effluent quality, better disinfection capability, less land requirement, higher volumetric loading and less sludge production (Judd, 2016). Since the membrane modules in an MBR are effective for biomass retention, the mixed liquor suspended solid (MLSS) concentration can be increased by 10 times comparing with CAS (Mori et al., 2006). This implies not only higher degradation rates, but also serious membrane fouling by solid deposition and, as a consequence, higher cleaning requirement. This is still a vital obstacle for their large-scale applications (Krzeminski et al., 2017), and in the past last decade, big efforts have been made by the scientist community searching for highly-efficient approaches to solve this problem.

Commonly used strategies include chemical and mechanical cleaning, optimization of operating conditions and pretreatment of the influent (Meng et al., 2017). Many factors have been verified to

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influence membrane fouling, including the interfacial interactions between membrane modules and foulants (Cai et al., 2017; Chen et al., 2017b), calcium ions (Zhang et al., 2018) and natural organic matter (Wang & Benjamin, 2016). However, new developments are based on the characteristics of the activated biomass contained in MBRs (Jorgensen et al., 2017), and, changing the growing status of biomass within bioreactor is one of the most recent mitigation proposals (Chen et al., 2016; Iorhemen et al., 2017). For instance, according to Sajjad et al. (2016), seeding granular sludge into an MBR decreased by 8 times of the membrane fouling compared to a conventional process. Furthermore, it was demonstrated that the MBRs with small floc size sludge would significantly increase the filtration resistance of membrane module, particularly the hydraulic cake resistance and osmotic pressure-induced resistance (Shen et al., 2015).

The above mentioned granular sludge is a collective of numerous self-immobilized microbial cells, which was first discovered in a strictly anaerobic system in 1980 (Lettinga et al., 1980), while the formation and application of aerobic granular sludge (AGS) has been studied since 1990 (Beun et al., 1999; Morgenroth et al., 1997). Currently, it is characterized by well-defined shape and structure, high settling velocity, enhanced microbial functions, resilient to toxicity and good ability for treating both industrial and domestic wastewater (Sarma et al., 2017; Zhang et al., 2016). Both of them can be successfully cultivated in up-flow anaerobic sludge bed reactors (Chong et al., 2012; Lim & Kim, 2014) and in sequencing batch reactors (SBRs) (Isanta et al., 2013) where the main parameter influencing sludge granulation is easy control. The latter has been regarded as the most successful way to cultivate AGS in a lab- and pilot-scale bioreactor (Isanta et al., 2012; Ni et al., 2009). However the high ratio of height to diameter (H/D) actually limits their application in full-scale plants, contrary to continuous-flow reactors which require lower installation costs and easier operation, maintenance and controlling (Juang et al., 2010).

Li et al. (2005) first presented a novel combination of MBR with AGS which resulted in advantages to effectively control membrane fouling without any negative effects during wastewater treatment. That was the starting point of the following experiments that proved MBR inoculation with mature AGS from an SBR was a promising solution to mitigate the problem. Thus, a continuous-flow reactor was previously operated with internal circulation in order to be the substitute of the feast/famine regime in SBRs for cultivating AGS directly. The research demonstrated the formed AGS were kept stable for more than 100 days and as a consequence their feasibility of self-cultivating in those reactor-types (Chen et al., 2017a). Moreover, to expedite the process, an enhanced internal-circulation MBR (EIC-MBR) was designed and continuously operated for more than 3 months (Wu et al., 2017), where diverse AGS co-existed.

This work presents a further investigation of the above mentioned system. Since it is possible to cultivate diverse AGS with a sole carbon source, and that might be beneficial to the microbial biodiversity promoting the bioreactor stability; the present work is mainly focused on the two unsolved issues, namely the heterogeneity of the self-cultivated AGS and their essential forming mechanisms in a single EIC-MBR. By implementing multi-methods, including wet density measurement, scanning electron microscopy (SEM), fluorescence microscope (FM) and high-throughput sequencing (HTS), the above mentioned heterogeneity was successfully illustrated.

2. Materials and methods

2.1. Experimental procedure and the operational conditions

An EIC-MBR operated in a continuous mode was used to cultivate the diverse sludge granules directly and operated for more than 3 months without discharging any excess sludge, except for sampling a small amount (100 mL) for analysis. The bioreactor was divided into an aeration and a mixing zone, its detailed configuration, controlling devices, internal circulations, inoculated sludge and the glucose-based synthetic wastewater were described in the previously published report (Wu et al., 2017). A hydrophilic polyvinylidene fluoride hollow membrane module (MOF-1d, effective area: 0.5 m^2, pore dimension: 0.2 μm, Tianjin Motianmo Membrane Technology Co., LTD, Tianjin, China) was installed in the aeration zone. The daily average dissolved oxygen (DO) concentration is calculated from data measured by two automatic detectors (JPSJ-605F, INES (Group) Co., Ltd, China) working in both zones every 60 s and recorded from 0:00 to 24:00. This paper mainly focused on sludge granulation from day 1 to day 115 and the long-term operation of the bioreactor and the characteristics of membrane fouling during the experimental period will be reported in another investigation.

2.2. Measurement of granule size and its distribution

To verify the sludge granules formation and their stability within the bioreactor, samples were collected from both zones to measure their granule size and its distribution on day 40 and 102 respectively, by using a laser particle size analyzer (Mastersizer 2000, Malvern Instruments Ltd., UK). For comparisons, the inoculated floc sludge was also sampled at the beginning and under the same conditions.

2.3. Analysis of the wet density of sludge granules

Specific gravity of the granules, also called wet density, was calculated according to Eq. (1).

$$\gamma \Big/ [\mathrm{T}\ ^{\circ}\mathrm{C}/4\ ^{\circ}\mathrm{C}]\,(\mathrm{g/cm^3}) = \frac{\mathrm{M_2}-\mathrm{M_0}}{[(\mathrm{M_2}-\mathrm{M_0})-(\mathrm{M_3}-\mathrm{M_1})/\rho]} \times \mathrm{F} \quad (1)$$

where γ is the wet density (g/cm^3) at T °C; M_0 is the weight of vacant centrifuge tube (g); M_1 is the weight of centrifuge tube filled with distilled water (g); M_2 is the weight of centrifuge tube with granules but discarding the supernatant after a 5-min centrifugation at 3000 rpm (g); M_3 is the weight of centrifuge tube filled with granules and distilled water (g); ρ is the density of water at room temperature (g/cm^3); F is the temperature correction factor (0.9982 at 20 °C).

2.4. Sludge granules observation by SEM

2.5. FM observation

Extracellular polymeric substances (EPS), common products from the metabolic activities of microorganisms, generally form a gel-like network in sludge granules that play an important role in keeping their stability (Cai et al., 2017; Hong et al., 2017). Thus, the *in-situ* distribution of EPS in the formed granules was observed by an FM method. Before each observation, the chosen sludge granules were pretreated by combining an enzyme hydrolysis and staining. Each sample was equally divided into four groups (Group A-D), among which, Group B-D were hydrolyzed by proteinase K (5000 U/μL in PBS), lipase (5 U/μL in PBS) and β-Amylase (5.13 U/μL), respectively. The tubes containing the granules and enzymes were shaken in a thermostatic water bath oscillator (SHA-B, Changzhou Aohua Instrument Co., LTD, China) at 150 rpm for 60 min at 37 °C. Meanwhile, Group A, as a control group, was just shaken directly in the tubes without enzyme hydrolysis and then stained. The procedures of enzyme hydrolysis were adopted by citing a published reference (Adav et al., 2008) but with some adjustments with the details described below: (1) Proteinase K (P109033, Aladdin, Shanghai, China), a stable and highly reactive serine protease with the ability of cleaving peptide bonds at the carboxylic sides of aliphatic, aromatic, or hydrophobic amino acids, was used for protein hydrolysis tests; (2) an enzyme which could hydrolyze fat into fatty acid and glycerinum – lipase (beef insulin, L111237, Aladdin, Shanghai, China) was utilized for lipase hydrolysis tests; (3) β-Amylase (from soybean, A0448, Tokyo Chemical Industry CO., LTD, Japan) was used

to break down the β-linkages in polysaccharides.

The control and hydrolyzed granules were stained by using the following scheme, namely, fluorescein isothiocyanate (FITC) (F104848, Aladdin, Shanghai, China) was used to stain proteins and amino-sugars, while the Nile red (N121291, Aladdin, Shanghai, China) was utilized to stain lipids, and calcofluor white (Sigma-Aldrich Co. LLC, USA) was applied to stain β-D-glucopyranose polysaccharides.

In the staining step, FITC solution (10 mg/L in dimethyl sulfoxide) was first added to the samples with a one-hour standing. After washing for three times with PBS, the samples were stained with calcofluor white (1 g/L) for 10 min. Then the samples were incubated with the Nile Red solution (10 mg/L in methanol) for 10 min. Finally, a drop of antifade solution (C1210, Applygen Technologies Inc, Beijing, China) was added into the samples. The stained samples were immediately observed by using an FM (Axioplan 2, Carl Zeiss Aktiengesellschaft, Jena, Germany). The parameters of the FM observation were set by using the control group as a contrast, and did not change until the last image of the same group was taken.

2.6. Analysis of microbial community

HTS was used to analyze the structure and the microbial community of the formed granules (pre-separated with a 0.2 mm-sieve) and the floc sludge (FS, < 0.2 mm) collected simultaneously. The details of HTS were described in the previously published short communication (Wu et al., 2017). Amplicon sequencing of 16S rDNA was adopted in this experiment to reveal the composition of microbial communities and the discrepancy among samples. In particular, samples were collected and then preserved in drikold before they were sent for DNA extraction, PCR amplification and sequencing analysis by the Illumina HiSeq sequencing platform of the V3-V4 region of 16S rDNA gene (Novogene Corporation, Beijing, China). For the convenience of comparison, the samples for all analyses of microbial community were collected on the same day (day 113).

After sequencing analysis, the raw reads were processed through UCHIME Algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) and Gold database (http://drive5.com/uchime/uchime_download.html) and a total of 64,813, 64,988, 62,069, 67,147 and 65,916 effective tags in the samples were obtained, respectively. Then these effective tags were classified into operational taxonomic units (OTUs) at the identity of 0.97 by Uparse (Uparse v7.0.1001, http://drive5.com/uparse/). Finally, OTUs were input in the commonly used MOTHUR software (http://www.mothur.org) to carry out species annotations based on the SSUrRNA database (SILVA, http://www.arb-silva.de/). Those annotation results contained several levels, including the phylum, class, order, family and genus level.

2.7. Determination of the flow patterns in both zones of the bioreactor

Reynolds number (Re), a dimensionless hydrodynamic variable to express the flow pattern at different situations, was used to determine that of the mixing and the aeration zone in the studied bioreactor. For the convenience of calculation, the reactor was simplified into two parts, including a cylindrical agitation tank with a screw propeller mounted in the middle (the mixing zone), and a tank with an aerator mounted in the bottom. The calculation methods of these two parts are listed below.

Re of the mixing zone (Re_m) was calculated with the following equation:

$$Re_m = \frac{\rho N D^2}{\mu} \quad (2)$$

where ρ (kg/m^3) is the density of MLSS in the mixing zone (measured by a standard method); N (r/s) and D (m) is the rotation speed and the characteristic diameter of the screw propeller, respectively; and μ (Pa·s) is the dynamic viscosity of the MLSS in the mixing zone (measured by a viscometer with an ultra-low viscosity adapter (DV-III ULTRA, Brookfield, Massachusetts, USA).

Re of the aeration zone (Re_a) was calculated with the following equation:

$$Re_a = \frac{\rho D U_L}{\mu} \quad (3)$$

where ρ (kg/m^3) is the density of the MLSS in the aeration zone; D (m) is the characteristic length of the tank; U_L (m/s) is the superficial liquid velocity; and μ (Pa·s) is the dynamic viscosity of the MLSS in the aeration zone.

U_L was calculated with the following equation:

$$U_L = 1.18(gDU_G)^{\frac{1}{3}} \quad (4)$$

where g (m/s^2) is the gravitational acceleration; D (m) is the characteristic length of the tank; and U_G (m/s) is the superficial gas velocity.

U_G is determined by the volume of gas flow from the following equation:

$$U_G = \frac{V}{A} \quad (5)$$

where V (m^3/s) is the gas flow volume in the aeration zone, and A (m^2) is the base area of the tank. The used parameters for calculation are listed in detail in SI.

3. Results and discussion

3.1. Granule size and its distribution

AGS could be clearly observed with naked eyes after operating for only 36 days. The results of granule size and its distribution on day 40 and 102 are shown in Fig. 1.

As shown in Fig. 1, an obvious difference in granule size and its distribution was observed between the inoculated and the granule samples. The size of the former was much smaller than that of the latter. Most of the former ranged from 0.05 to 0.08 mm, while the latter had a comparatively larger size ranging from 0.2 to 0.6 mm. And the size of most of the latter was larger than 0.2 mm, which meant that the sludge granules with larger size occupied a relatively higher ratio within the bioreactor. Furthermore, no much difference between the granule size and its distribution on day 40 and 102 indicated the sludge granules were kept stable during the observed period.

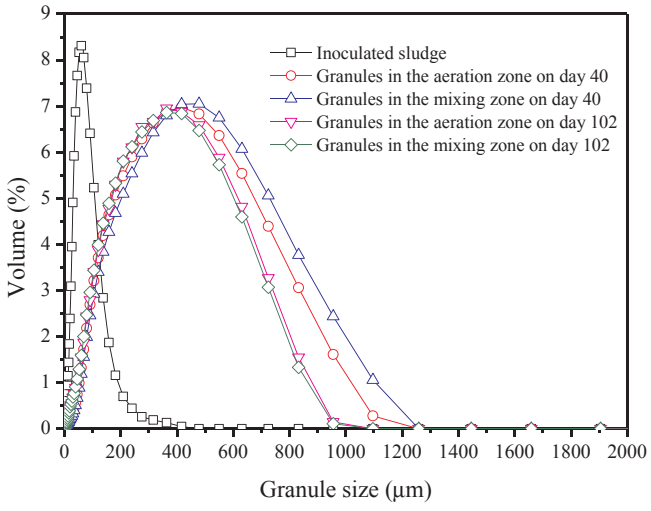

Fig. 1. Granule size and its distribution.

3.2. Observation of the diverse sludge granules

The observation of diverse sludge granules actually lasted for over one year. However, this paper has mainly focused on the stable period in terms of COD and TN removal (i.e. from the 1st to 115th day where the average was over 85% and 60%, respectively). Floc sludge was inoculated into the bioreactor and after only operating for 36 days, sludge granules with clear edge were found to form in the bioreactor. As the extension of time, these sludge granules grew to mature and gradually showed diverse colors in their appearance. About operating for 113 days, the formed AGS showed at least four types of color within the bioreactor (shown in the attached Electronic Annex, Still images). They were sampled and named by their colors, that is, Black Granular Sludge (BGS), Yellow Granular Sludge (YGS), White-margin and Black-middle Granular Sludge (WBGS), and Black-margin and Yellow-middle Granular Sludge (BYGS). Apart from the different colors in their appearance, their shape also showed a significant difference. BGS exhibited spherical or ellipsoidal shapes with quite an obvious variation in their size. YGS showed much regularized morphology with an ellipsoidal shape. WBGS had a flat shape with thicker-black-interior structure, and BYGS also had a flat shape, but with thinner-yellow-interior structure.

Barr et al. (2010) once found two distinct granule types (white and yellow) co-existed in an SBR during the initial stage of granulation (i.e. day 58 to 98). However, as the experimental time extended to day 133, those disappeared and instead, a single homogeneous off-white appeared. Based on this observation, they proposed the formation mechanism for these two types of sludge granules and also gave an explanation for their disappearance at the initial stage. In the presented experiment, it was also observed the variation of the self-cultivated diverse sludge granules in their color, but from a long-term observation (more than a year), diverse AGS with different colors were still found to be co-existing in the bioreactor. Finally, the above mentioned different colors found in diverse granules suggest their heterogeneity given by different chemical and microbial composition, and thus, the need for further analysis by SEM observation.

3.3. SEM observation

The obvious different colors of the selected sludge granules in their appearance imply that the self-cultivated AGS may exhibit heterogeneity in their basic characteristics. The results of SEM observation are shown in SI. The results indicated that all kinds of granule sample had a clear edge and filamentous bacteria surrounded each granule to maintain the structural integrity and stability for zoogloea cohering. Some tunnels could be observed in each sample, especially in YGS, whose function might be transferring nutrient and oxygen. BGS seemed to have a more compact structure, and YGS had more pores and *Bacillus*. The black part of both BYGS and WBGS was more compact than the yellow part, and also a white part could be observed. In the white part, lots of filamentous bacteria were found, and while, in the black part, almost no filamentous bacteria were observed. From the SEM observation, it could be summarized that every kind of granules showed heterogeneity in their micro-structure.

3.4. Wet density

By using the methods described in Section 2.3, the wet density was measured and is listed in Table 1.

Table 1
Wet density of the four types of sludge granule.

Samples	BGS	WBGS	YGS	BYGS
Wet density (g/cm^3)	1.86	1.37	1.31	1.09

The wet density of all samples shown in Table 1 is all larger than 1 g/cm^3. There is an obvious difference between BGS and the other samples, which imply that BGS may have a more compact structure and the result of SEM confirms that (see SI).

3.5. EPS distribution in the sludge granules

The most appealing merit of FM observation is its *in-situ* showing of the visual distribution and relative content of different EPS on the observed granules. As shown in the results, proteins, lipids and β-polysaccharides were observed to distribute mainly at the outer rim of BGS, while around YGS, proteins, lipids and β-polysaccharides distributed erratically. The bright-field photographs showed that BYGS and WBGS both had a heterogeneous structure, in which, BYGS showed a dark outer margin and a light middle, and WBGS exhibited a dark middle and a light margin. Such an observation supported the heterogeneity of BYGS and WBGS obtained from their SEM results. Based on the fluorescent intensity in the results, there were more lipids and β-polysaccharides in the yellow part than that in the black part, while proteins distributed evenly around BYGS. In the black part of WBGS, it showed that there were more proteins, lipids and β-polysaccharides in the white part than that. From the above results, it could be verified that the white contained the most proteins, lipids and β-polysaccharides, then the yellow contained the second, and the black contained the least.

The above results also indicated that even after the enzyme hydrolysis, the granule samples still kept their stable structure and integral morphology, which demonstrated that the filamentous-bacteria-skeleton played an important role in granule formation and stability.

3.6. Composition of the microbial community in the sludge granules

The results of HTS are shown in Table 2 and Fig. 2. The table shows three indexes which have different meanings. The first (see the fourth column, Table 2), is the coverage of each clone library and a measure of the captured diversity which reflects the reliability of the sequencing results, and a higher value indicates fewer non-captured diversity. Goods coverage of every sample is over 0.997, which means the sequencing results are extremely reliable. On the other hand, both Shannon and Simpson indexes are closely related to the richness and evenness of a community. Shannon index is used to quantify the entropy in the strings of text, whose high value indicates a higher diversity. The Simpson index measures the degree of concentration when individuals are classified into several types, whose values shown in Table 2 represent the probability that two individuals randomly selected from a sample belonging to different species and the larger value indicates a higher diversity. Table 2 shows that both Shannon and Simpson index ranking from the higher to the lower order of all samples are BGS, YGS, FS, BYGS and WBGS with the same sort order of diversity, which indicates that BGS has the highest diversity and WBGS has the lowest.

For further revealing the microbial communities contained in these sludge granules and their relationship, the HTS results are shown in Fig. 2.

Fig. 2(A) showed the similarity and difference of the OTUs among all the samples, which indicated that quite a large amount (629) were

Table 2
Effective tags, OTUs and three alpha diversity indexes.

Sample name	Effective tags	OTUs	Goods coverage	Shannon index	Simpson index
FS	64,813	1229	0.997	6.303	0.944
BGS	64,988	1351	0.997	7.307	0.979
BYGS	62,069	1211	0.999	6.187	0.926
YGS	67,147	1288	0.997	6.843	0.964
WBGS	65,916	1122	0.997	5.116	0.836

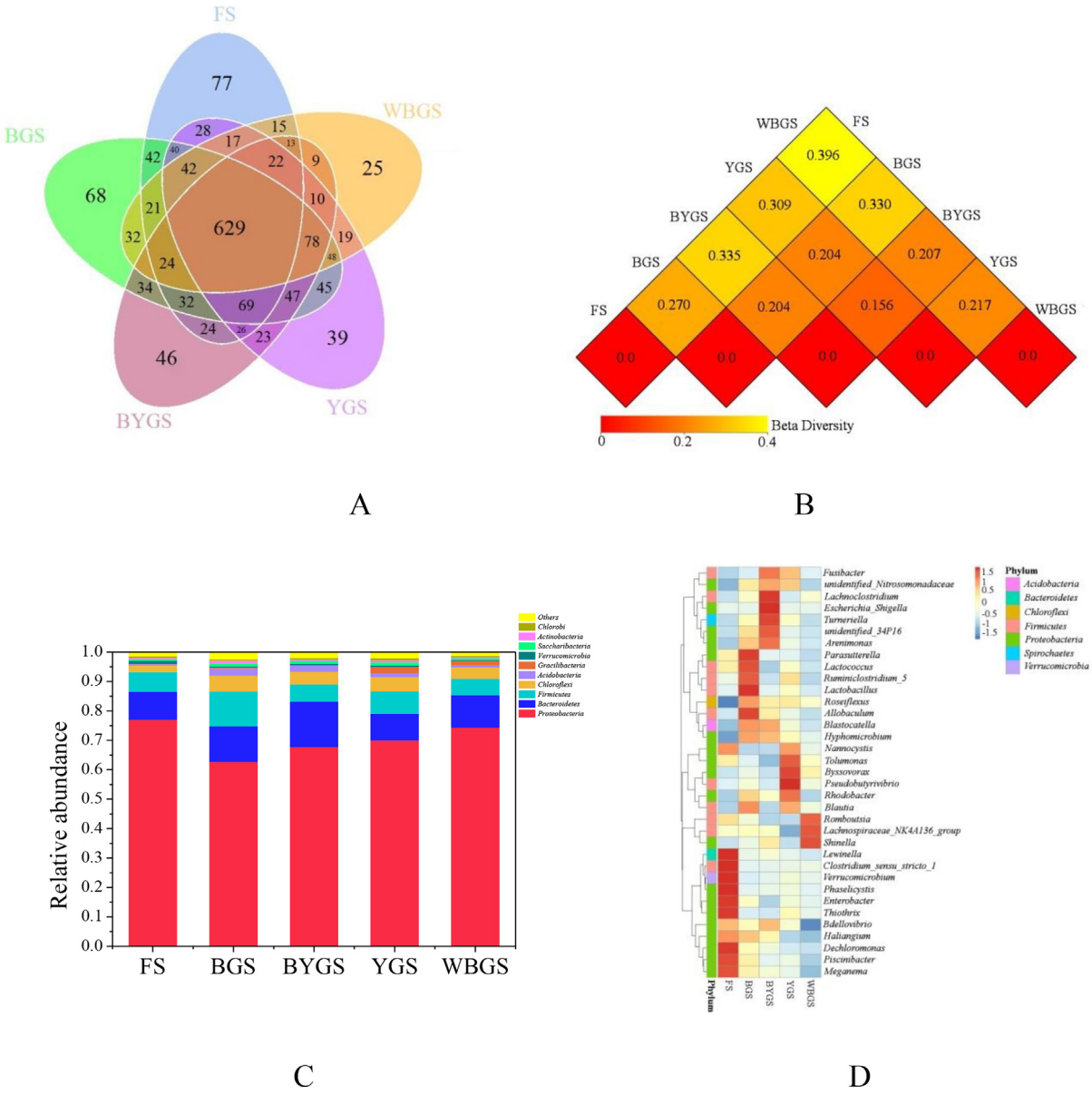

Fig. 2. The results of HTS: (A) Venn diagram; (B) Beta diversity heat map on weighted UniFrac; (C) Top-10 relative abundance at the phylum level; (D) Abundance heat map at the genus level.

shared for all the five samples. And every sample had its own unique OTUs, among which, FS contained the most, but WBGS contained the least.

Beta-biodiversity generally refers to the response of organisms to spatial heterogeneity, and the high value implies a low similarity between the microbial species composition of different communities. Fig. 2(B) showed the heat map of beta-diversity based on the weighted UniFrac, in which, FS showed the most obvious difference with that of other samples, and the value between YGS and BYGS was the lowest, which indicated that they had the most similar composition. Nevertheless, among the obtained values of beta-biodiversity, various degrees of difference indicated that they were quite obviously different in the composition of microbial communities.

Fig. 2(C) further revealed the relative abundance of microorganisms at the phylum level, which showed that, *Proteobacteria* was the dominant group among all the five samples, whose ratios in FG, BGS, BYGS, YGS and WBGS were 76.89%, 62.64%, 67.67%, 69.92% and 74.34%, respectively. In general, *Proteobacteria* was a group of Gram-negative bacterium, whose major components on their outer surface were bacterial lipopolysaccharides, which made these bacteria easier to attach on the surface of carrier (Atabek & Camesano, 2007; Charnock & Nordlie, 2016). With these cohesive substances, suspended sludge particles could aggregate together. In this meaning, *Proteobacteria* might be regarded as a kind of key group bacteria to help other microorganisms and sludge particles agglomerating together. The sub-dominant phylum among all the samples was *Bacteroidetes*, with the ratios of 9.55%, 12.12%, 15.51%, 9.12% and 11.00% in FS, BGS, BYGS, YGS and WBGS, respectively. These dominant and sub-dominant compositions at the phylum level consisted with other reports (Duan et al., 2015; Guanglei Qiu et al., 2013). *Bacteroidetes* were composed of three large classes of Gram-negative bacteria, including non-spore-forming, anaerobic or aerobic, and rod-shaped bacteria, which were widely distributed in the environment. The third largest phylum among all the samples was *Firmicutes*, occupying 6.73%, 11.85%, 5.82%, 7.64% and 5.61% of FG, BGS, BYGS, YGS and WBGS, respectively. *Firmicutes*, most of which are Gram-positive bacteria, were generally found to be the

major bacteria in some anaerobic bioreactors (Takashi Narihiro et al., 2009). Their occurrence in all the samples, especially their ranking at the top-3 group in the samples, verified that an anaerobic habitat did exist in these granule samples.

At the phylum level, the main observed phyla seemed similar in all the samples even they had a different occupying ratio in all the detected phyla. However, significant differences among the samples in their microbial community were observed at the genus level (Fig. 2(D)), in which, *Phaselicystis*, *Enterobacter*, *Thiothrix*, *Dechloromonas*, *Piscinibacter*, *Meganema* (belonging to *Proteobacteria*), *Lewinella* (belonging to *Spirochaetes*), *Clostridium sensu stricto 1* (belonging to *Firmicutes*) and *Verrucomicrobium* (belonging to *Verrucomicrobia*) were the dominant species in FS, but most of which were almost not found in other samples. *Enterobacter* was a genus of common Gram-negative, facultative anaerobic, rob-shaped, non-spore-forming bacteria, and *Enterobacter cloacae CF-S27* was found to have a special function of simultaneous nitrification and aerobic denitrification in the presence of high concentration of hydroxylamine, as well as having a tremendous potential to produce bacterial floc (Padhi et al., 2017). *Thiothrix* was a genus of filamentous sulfur-oxidizing bacteria, and it had the potential for the biological removal and recovery of phosphate and sulphur (Rubio-Rincon et al., 2017). While in BGS, the prominent species were *Parasutterella* (belonging to *Proteobacteria*), *Lactococcus*, *Ruminiclostridium 5*, *Lactobacillus* and *Allobaculum* (belonging to *Firmicutes*). *Parasutterella*, a genus of Gram-negative, strictly anaerobic, non-spore-forming bacteria, was found in abundance in BGS but marginal in other samples. The dominant species in BYGS included *Escherichia Shigella*, *Arenimonas* (belonging to *Proteobacteria*), *Fusibacter*, *Lachnoclostridium* (belonging to *Firmicutes*) and *Turneriella* (belonging to *Spirochaetes*). *Escherichia Shigella*, a genus of Gram-negative, facultative anaerobic, non-spore-forming, rob-shaped bacteria, glucose-decomposing (Yabuuchit, 2002), was only observed in BYGS. The detected *Fusibacter fontis*, a species of genus *Fusibacter*, was a sulfur-reducing and anaerobic bacterium (Fadhlaoui et al., 2015). *Tolumonas*, *Byssovorax*, *Rhodobacter* (belonging to *Proteobacteria*) and *Pseudobutyrivibrio* (belonging to *Firmicutes*) were the dominant species in YGS. *Pseudobutyrivibrio*, a genus of Gram-negative, anaerobic, non-spore-forming, butyrate-producing and flagellum-motile bacteria (Kopecny et al., 2003), was only found in YGS. *Romboutsia*, *Lachnospiraceae NK4A136 group* (belonging to *Firmicutes*) and *Shinella* (belonging to *Proteobacteria*) were the dominant species in WBGS, which were not particular among other samples. *Romboutsia* was a genus of Gram-positive, rod-shaped, non-mobile, spore-forming obligate anaerobic and acetate-producing bacteria (Gerritsen et al., 2014), whose anaerobic feature verified the existence of an anaerobic condition inside WBGS. *Shinella* was a genus of Gram-negative, non-spore-forming and motile-robs bacteria, which generally grew in liquid media with an amorphous or finger-like flocculent (An et al., 2006) and was consistent with the SEM observation of WBGS.

The above results indicated that the dominant species and microbial composition at the genus level were quite different among the four granule samples, which demonstrated that their essential properties showed an obvious heterogeneity. However, different kinds of anaerobic microorganisms were found in all the granule samples, and further verified the existence of anaerobic condition inside each kind of granules. Microbial heterogeneity existed in every kind of granules and thus created a rich habitat in the bioreactor, which were in favor of the granule stability of long-term operation in the bioreactor. Interestingly, *Roseiflexus* (belonging to *Chloroflexi*), a Gram-negative, thermophilic and anoxygenic filamentous phototrophic bacterium (Satoshi Hanada et al., 2002), was not found in FS, but found in the other four granule samples. Based on the above findings, it could be concluded that this kind of filamentous bacterium played an important role in keeping the stability of granules.

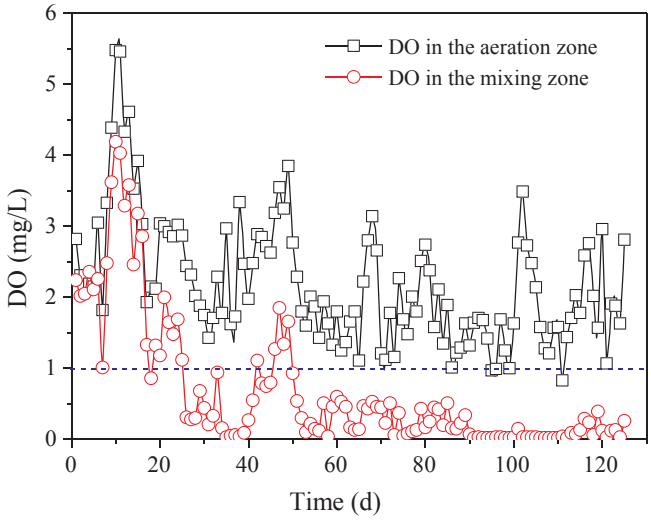

Fig. 3. DO distribution in the aeration and the mixing zone within the reactor.

3.7. Essential mechanism causing heterogeneity of the granular sludge

3.7.1. The effect of dissolved oxygen

DO is a limiting factor to define an anaerobic/aerobic aqueous environment and, as a consequence, the occurrence of dominant microbial species. In a previously published report (Tang et al., 2014), it was verified that a multi-habitat could be gradually formed as the formation of an obvious DO difference within an MBR. The present experiment also measured the DO distribution at different zones within the bioreactor, and the results are shown in Fig. 3.

As shown in Fig. 3, the DO concentration shows an obvious difference between both zones considered, i.e. relatively higher values where the air is being sprayed directly and it was constantly kept up to 1.0 mg/L during the whole operational period. On the other hand, the remnant DO was transferred by the internal circulation to the mixing zone. Along with the operation time, the biomass in the whole bioreactor increased gradually which caused a sharp and quick decline of the oxygen content in the mixing zone immediately after start-up.

Obviously, the different DO concentration at different zones causes quite diverse dominant microbial species distributing within the bioreactor, which provides various initial seed microorganisms at different zones for sludge granulation.

3.7.2. The effect of internal circulation

Based on the basic assumptions and the equations described in Section 2.7, it could be calculated that Re_m and Re_a were 5.829 and 27.776 in both zones, respectively. Since they were under Re 2000, they were characterized by a laminar regime. In an agitation tank, Re_m smaller than 10 means that the area near the screw propeller is a laminar flow, but the other parts far from it are stagnant areas. Though the fluids are all in a laminar status, Re_a is about five times as much as Re_m, such a difference between Re_m and Re_a implies different degrees of fluid turbulence existing in this single bioreactor. In this way, the biomass transferring between the two zones may be granulated by the different hydrodynamic shear stresses, which gradually forms sludge granules.

Additionally, apart from a large internal circulation between the aeration and mixing zone, lots of tiny eddies (Videos 1–3) were also observed distributing in the bioreactor. Video 1 showed an integrated internal circulation occurring between the aeration zone and the mixing zone, and Video 2 revealed some small swirls formed in the aeration zone, and Video 3 exhibited a clockwise circulation in the mixing zone.

The above complex internal circulation caused the granulation occurring everywhere.

Supplementary Video 1.

The micro-habitat in a bioreactor directly determines the structure and dominant species of microbial communities. However, many factors heavily influence its formation, including the inlet carbon and nutrient sources, the DO concentration, the reactor configuration and its hydrodynamic conditions. Recent studies have verified that different substrates could create a different constitution in the microbial community and form diverse sludge granules in different SBRs (Figueroa et al., 2015). However, in a single MBR, it is a novel phenomenon reported previously and published in a short communication (Wu et al., 2017), finally, its heterogeneity was further revealed by the presented experimental results. On the other hand, in a biofilms the above mentioned heterogeneity might be triggered by different local conditions, as was typical for the growth and development of microorganisms in spatially heterogeneous ecosystems (Singer et al., 2010). However, as mentioned in Section 3.7.1, in the presented bioreactor, it actually formed a spatially heterogeneous ecosystem or a multi-habitat within a single apparatus. Based on the aforementioned experimental results and analysis, the mechanism of causing the heterogeneity of diverse sludge granules in the presented bioreactor can be summarized as follows and illustrated by the schematic diagram in Fig. 4:

Supplementary Video 2.

Supplementary Video 3.

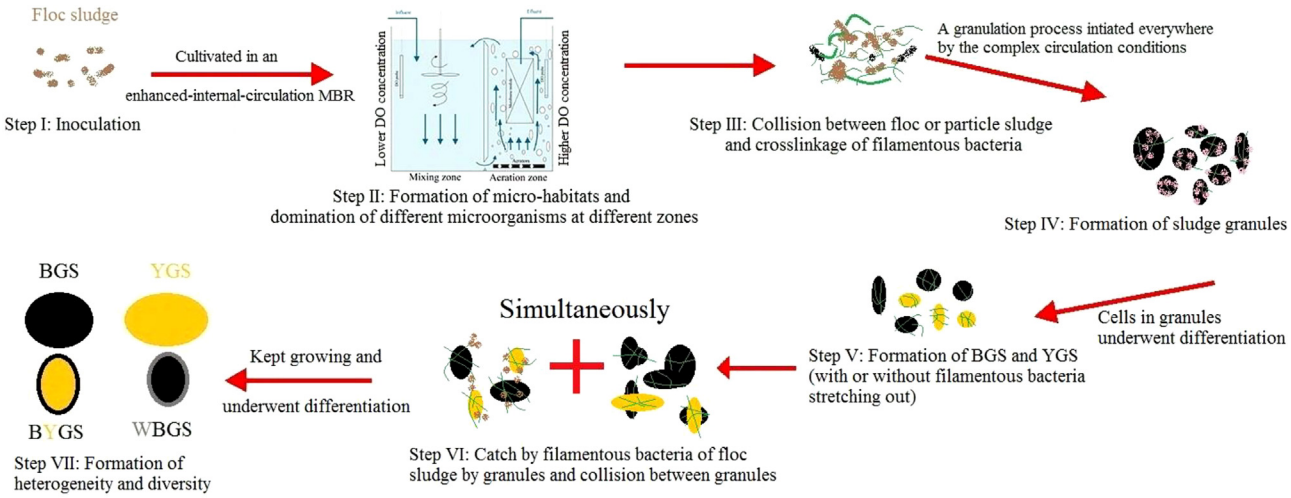

Fig. 4. Schematic diagram for the mechanism followed by the studied self-cultivated diverse sludge granules.

1) Micro-habitats created by different DO conditions led to some microorganisms dominate at different zones, which provided different microbial species for the following granulation process;
2) The contained filamentous bacteria cross-linked floc or particle sludge and provided suitable places for the aggregated bacteria to colonize on;
3) Complex circulation conditions initiated a granulation process everywhere in the bioreactor;
4) The aggregated biomass was kept circulating by the internal circulation and secreted EPS simultaneously, which made it possible to become compact under the hydraulic shear force;
5) The formed sludge granules became relatively independent communities under the protection of the outer EPS shell, which gave them a chance to continue their succession process and gradually exhibited an obvious heterogeneity in their physico-chemical and microbiological characteristics.

4. Conclusion

Diverse AGS were successfully cultivated in a continuously operated EIC-MBR with a sole carbon source. They showed high bio-activity and heterogeneity in their basic physic-chemical and microbial characteristics, in which, filamentous bacteria acted as an important skeleton to maintain the integrity. Having implemented multi-methods, it was verified that the micro-habitats created by an enhanced hydrodynamic condition along with the intertwining of sludge floc by filamentous bacteria and the pressure of hydraulic shear force among the granules, were the direct reasons causing the heterogeneity of diverse sludge granules within the bioreactor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.05.004.

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